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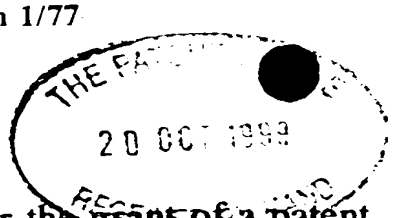

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REP05981GB

2. Patent application number
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9924880.9

20 OCT 1999

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Cambridge University Technical Services Ltd
The Old Schools
Trinity Lane
Cambridge
CB2 1TS
United Kingdom

Patents ADP number *(if you know it)*

6956809001

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

See continuation sheet for further applicant(s)

4. Title of the invention

OPTICAL MICROSCOPY AND ITS USE IN THE STUDY OF CELLS

5. Name of your agent *(if you have one)*

GILL JENNINGS & EVERY

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
7 Eldon Street
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EC2M 7LH

Patents ADP number *(if you know it)*

745002

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Number of earlier application	Date of filing *(day / month / year)*

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11. For the Applicant
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature

Date
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12. Name and daytime telephone number of person to contact in the United Kingdom

PERRY, Robert Edward
0171 377 1377

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Patents Form 1/77 **Continuation Sheet**

ur reference: REP05981GB

Applicant Details

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Country/State of incorporation: United Kingdom

OPTICAL MICROSCOPY AND ITS USE IN THE STUDY OF CELLS

Field of the Invention

This invention relates to optical microscopy and its use in the study of cells.

Background of the Invention

The cell is the most fundamental unit of living organisms, whether animal or plant. The study of its structure and composition, and how its various constituents function, lends valuable insight into the complex processes that occur in integrated biological systems. This requires techniques that allow investigation of cell samples to be conducted in real-time, non-invasively, and in solutions that mimic physiological conditions so that cell functionality is retained.

Optical microscopy (using visible light) has been widely applied to study live cells. However, the resolution is limited by diffraction to about 200-250 nm. For more detailed study, one commonly used method is electron microscopy, where it is possible to obtain images with 10 nm resolution, but the sample needs to be fixed prior to imaging. Hence, it is not possible to use an electron microscope to study living cells.

Another possible high resolution technique is based on the use of scanning probe microscopy (SPM), in which a sharp probe tip is scanned in close proximity to the sample under study. The consequent interactions and thus the chemical/physical properties of the sample can be plotted as a function of the tip's position with respect to the sample, to generate a profile of this measured interaction. Members of the SPM family that are commonly applied to biological imaging are atomic free microscopy (AFM), scanning ion-conductance microscopy (SICM) and scanning near-field optical microscopy (SNOM).

In SNOM, light is normally coupled down a fibre-optic probe with an output aperture of sub-wavelength dimensions, which is scanned above the sample surface. Interaction forces between the tip and sample are used to maintain their separation at less than the sub-wavelength dimensions of the aperture. This arrangement allows simultaneous generation of optical and topographic images whose resolution depends on the size of the output aperture and the size of the tip respectively. As in far field optical microscopy, all contrast mechanisms are available in SNOM, and in particular chemical imaging is possible by the use of fluorescent labels. However, while it is straightforward to fabricate probes with smaller apertures, achieving smaller tip-sample separations in

liquid (<60 nm) is difficult because of the problems in obtaining a reliable method of controlling the probe-sample distance. This is due to damping of the oscillations of the probe used in the feedback mechanism.

In SICM, an electrolyte-filled, glass micropipette is scanned over the surface of a sample bathed in an electrolytic solution; see Hansma *et al* (1989) Science 243:641-3. The pipette-sample separation is maintained at a constant value by controlling the ion-current that flows *via* the pipette aperture. The flow is between two electrodes: one inside the pipette and another outside in the electrolyte solution. For an applied bias between the electrodes, the ion-current signal depends on a combination of the micropipette's resistance (R_P) and the access resistance (R_{AC}) which is the resistance along the convergent paths from the bath to the micropipette opening. R_P depends on the tip diameter and cone angle of the micropipette, whereas R_{AC} displays complicated dependence on the sample's electrochemical properties, geometry and separation from the probe. It is R_{AC} that lends ion-current sensitivity to the pipette-sample separation and allows its exploitation in maintaining the distance such that contact does not occur.

The optimum tip-sample separation that has allowed SICM to be established as a non-contact profiling method for elaborated surfaces, is equal to one-half of the tip diameter; see Korchev *et al* (1997), J. Microsc. 188:17-23, and also Biophys. J. 73:653-8. The tip's output is used to generate topographic features and/or images of the local ion-currents flowing through pores on the sample surface. The spatial resolution achievable using SICM is dependent on the size of the tip aperture, and is typically between 50 nm and 1.5 μm. This produces a corresponding resolution.

Summary of the Invention

In order to meet the objective of high resolution microscopic study of cells that are alive, and not fixed, a hybrid scanning ion conductance and scanning near field optical microscope has been developed. Accordingly, in one aspect of this invention, apparatus comprises a probe *via* which an assay component may be delivered; a sensor to detect ion current; and means for controlling the position of the probe relative to the object in response to the ion current. In another aspect, a method for imaging an object in a liquid environment, by scanning ion-conductance microscopy, using a probe whose distance from the object is maintained in response to the ion current in the liquid, wherein the probe includes means for delivering an assay component to the object.

Use of the novel apparatus allows quantitative, high-resolution characterisation of the cell surface and the simultaneous recording of topographic and optical images. A particular feature of the method is a reliable mechanism to control the distance between the probe and the sample in liquid, e.g. physiological buffer.

The new method has been demonstrated by recording near field images of living cells (cardiac myocytes) for the first time. Straightforward modifications to the instrument will enable fluorescence imaging and higher resolution.

The present invention is based in part on a realisation that SICM and SNOM techniques are complementary (SICM as a non-contact profiling method and SNOM as a technique that allows acquisition of optical and chemical information pertaining to a sample) and that they can be used to advantage if they are in one experimental arrangement.

The invention allows functional mapping of cells. For example, it allows ion channel mapping.

By means of the invention, it is possible to image the cell surface in a single scan, by using the probe to keep the cell surface in the confocal volume of the microscope. For biological imaging of live cells, the exposure of light can be limited, thereby minimising damage and overcoming the problem of intense near field light sources used in SNOM. Although the cell may alter shape, this is not a problem in this surface confocal mode, since imaging is always at the surface.

A feature of this invention is its simplicity. For example, existing confocal microscopes can readily be retrofitted with a pipette and suitable computer control.

Description of the Drawings

A micropipette of the type that may be used in this invention is illustrated in Figure 1 of the accompanying drawings. Figures 2A and C illustrate how non-modulated ion current may be used to control probe position over a sample; Figure 2B illustrates how feedback control may be used, as in a preferred embodiment of this invention.

Description of the Invention

The term "assay component" is used herein to describe any chemical or physical entity that can be delivered to the locus of observation, and which is either observable *per se* or can generate an observable response. For example, the assay component may be light; a laser may be provided, so that, for example, coherent light can be directed, *via*

the probe, to a cell surface. Alternatively, possibly again in combination with a laser source, the probe may contain at its tip a material, such as a light-activatable dye, that will generate light *in situ*. The outer surface of the probe may be coated, e.g. with a metal layer, to prevent leakage of light.

Thus, for example, a micropipette is filled with one or more fluorophors and excited with a laser. In one case, the laser comes up the microscope objective; this produces a local light source since the laser is focussed at the tip of the pipette. In addition, the dye is concentrated, so that the depth of penetration of the laser light into solution is very small. The dye is under pressure and so slowly leaks out of the pipette, avoiding problems in photobleaching. This can be used to image in the near field; no metal coating is required.

In another embodiment, the assay component is a chemical reagent, and this reagent may act directly or indirectly. Examples of reagents that have a direct effect include those that generate fluorescence, bioluminescence or chemiluminescence. Thus, for example, a micropipette may be adapted to deliver luciferin that is acted on by luciferase in the presence of ATP and magnesium ions, to produce light peaking at 568 nm. Magnesium may be provided in the solution and all the other reagents in the pipette, so that light is produced locally by the reaction. No coating is needed to produce the near field light source. Rapid dilution of reagents, once they emerge from the pipette, means that the light is produced at the tip and the resolution is determined by the aperture of the pipette.

Other suitable reagents include molecules that change fluorescence with variation in a particular property such as pH, in concentration, e.g. of Ca, or potential. Local application of appropriate reagents and excitation of the fluorophor allows local probing of this property, e.g. map channel or proton pumps.

Examples of reagents that act indirectly are those that, on delivery to the cell, product a change inside the cell, as a result of transduction. It is that change which is detected. This effect may be naturally amplified by the signal cascade.

More specifically, the pipette may contain a ligand or drug. This acts on or binds a receptor on the cell surface. Inside the cell, as a result of the signal transduction cascade, there is a change in the level of a secondary messenger, for example in the cell's calcium level. There is amplification of the binding event by the cascade where an

enzyme is turned on, producing many product molecules which in turn act on many other enzymes etc., so that one binding event results in a large change. This naturally amplified signal is detected, for instance, using a fluorescence dye, e.g. fluor-3, which binds calcium in the cell, and can be used to measure calcium concentration. A large change in calcium can be seen when the pipette is over the receptor. Since calcium is the most common messenger used by the cell, this is a general method. It does not involve the use of fluorescently-labelled antibodies to detect where a receptor is located. Antibodies, particularly monoclonal, can be hard to produce and can have problems that they are internalised in the cell. Other fluorescence markers can be used for other common messengers in the cell, such as cAMP.

Fig. 1 shows a micropipette having a tip portion which is coated, e.g. with metal, to prevent leakage of light. The micropipette also comprises an optical fibre, *via* which laser light can be directed onto the sample to be tested. This is connected to a laser source (not shown).

The present invention may be illustrated by modification of an existing SICM system, to allow simultaneous generation of SICM and SNOM images of living cardiac myocyte cells. Cardiac myocyte cells were chosen for study for two main reasons. Firstly, they are composed of light and dark bands of material/striations that periodically occur every 2.1 μm which give them a distinct appearance and therefore make them a good model system for study. Secondly, and most importantly, they constitute heart muscle chambers that synchronously contract to produce the crucial pumping activity to circulate blood to the rest of the body. These cells have previously been studied using SICM (Korchev *et al*, *supra*).

The results presented here are the first images of live cells taken using scanning near field optical microscopy and show that scanning ion conductance microscopy provides a reliable control mechanism for SNOM imaging of live cells. The images of cardiomyocytes have the widely accepted structure and dimensions: comparable, for example, to those found with electron microscopy. By comparison, an important advantage of this invention is that the cells are unfixed and alive.

Although a simple hybrid SICM-SNOM instrument already has the potential to be a powerful investigative tool, straightforward modifications can be made, to improve its sensitivity and resolution. Firstly, smaller coated pipettes may improve both the SICM

and SNOM resolution and increase the size of the optical signals, by holding the near field probe closer to the sample. For example, high resolution SNOM probes may be made by tapering and coating a micropipette for imaging in air using force control (Harootunian *et al*, 1986). An optical resolution less than 100 nm was obtained. This indicates that fabrication of higher resolution near field probes for SICM-SNOM is feasible. Using a higher numerical aperture objective for collection of the light can further increase the optical signal. These improvements will also enable fluorescence imaging and also minimise problems in photobleaching and photodamage by working at minimum laser intensity. It is easier to photodamage living cells than fixed cells, so reducing the required laser power is important in order to be able to take multiple scans over time. These improvements in combination should enable simultaneous SICM and SNOM images with a resolution better than 100 nm on live cells, to be obtained.

In addition, if combined with fluorescence imaging, it may be possible to image receptors and channels on the surface of live cells and follow changes in the response to specific stimuli. Such an instrument would have a wide range of possible uses in biological science.

In particular, by using the ion current to control the distance between a coated micropipette and the sample, it is possible to obtain simultaneous optical and SICM images. The optical images are obtained in the near field and hence this appears to be reliable way to perform SNOM imaging of live cells and this is the first time this has been achieved. Straightforward improvements to the instrument should enable fluorescence imaging and higher resolution to be obtained. The new method of SICM-SNOM imaging of live cells has a wide range of possible applications in biological science.

In a preferred embodiment of the invention, a frequency-modulated scanning protocol for a scanning ion conductance microscope (SICM) is used. This may comprise means to vibrate a SICM probe in a vertical direction (Z) and to use this modulation for feedback control of the microscope. In previous SICM microscopes, non-modulated (I_{DC}) ion current (Fig. 2A and C) was used to control the probe position over the sample. In that case, any changes in ion current flowing through the micropipette tip which are not caused by the probe/sample interaction (changes in ion concentration, interaction of the micropipette tip with the particles contaminating aqua's solutions, drift of electrode potential, etc) produce artefacts or crash the sample and the micropipette. In the

frequency-modulated mode of SICM operation, the movement of the microscope tip (ΔZ) generates modulated current (I_{MOD}). This I_{MOD} is only generated when the probe senses the sample and is used for feedback control of the microscope; see Fig. 2B and C (insert). The feedback control mainly uses, for example, the frequency-modulated scanning protocol, as it has a number of additional advantages over a non-modulated mode: greater signal/noise ratio; high stability (ability to operate in a large gradient of electrolyte and with high I_{DC} drift); higher scan speed; increase in lateral sensitivity.

A typical SICM system consists of components that feature in all SPMs, namely, scanning probe, piezo-actuator scanning elements, control electronics and a computer. These components are built in and around an inverted microscope (Diaphot 200; Nikon Corporation, Tokyo, Japan) central to the experiment.

SICM probes may be fabricated by pulling borosilicate, glass microcapillaries with outer and inner diameters of 1.00 mm and 0.58 mm respectively, using a laser-based micropipette puller (Model P-2000, Sutter Instrument Co., San Rafael, CA, USA). This reproducibly and easily produces probes with conical taper lengths and apex diameters of 200 nm, 400 nm and 1.0 μm, respectively.

Three-dimensional and high precision movement of the probe relative to the sample is achieved by the piezo-translation stage (Tritor 100, Piezosystem Jena, Germany) on which the SICM probe is mounted. The stage has a range of 100 μm in the x, y and z directions so that scanning over biological samples, with features that scale up to 30-50 μm is made possible. The high voltage required for deformation of the piezo-ceramic material that facilitates the stage's movement is provided by high voltage amplifiers (Piezosystem Jena, Germany). These amplifiers respond to appropriate signals generated by the control electronics to drive the piezo-translation stage and achieve movement of the tip relative to the sample. In addition to being connected with the hardware aspect of the microscope, the control electronics interfaced with a computer that allows data acquisition and image analysis. The control/data acquisition hardware and software are produced by East Coast Scientific (Cambridge, UK).

The pipette-sample separation is maintained at a constant value by monitoring the ion-current that flows between Ag/AgCl electrodes in the micropipette and electrolyte solution in which the sample is immersed. Phosphate-buffered saline (PBS) solution is used for both filling the micropipette and the electrophysiological medium of the cardiac

myocytes so that concentration cell potentials and liquid junction potentials are not established . The ion-current is measured for DC voltages of 50 mV applied to the electrodes. It is amplified by means of a high-impedance operational amplifier (OPA129, Bull Brown International, USA) and converted to a voltage signal over a resistance of 10^8 Ω. This signal is then inputted into the control electronics where it is used for feedback control and data acquisition.

The micropipette is housed in a special, custom-made holder which is assembled together with the current amplifier and piezo-translation stage to comprise the SICM head. The SICM head is mounted onto the arm of the inverted microscope's z-translator that facilitates coarse vertical positioning of the micropipette relative to the sample positioned immediately below it. The sample is contained in a petri dish which is placed on the microscope's stage. Movement of the sample relative to the micropipette is achieved by the x,y translation controls of the stage. The processes of monitoring the vertical position of the micropipette relative to the sample and selection of an area of interest on the sample can be viewed on a TV screen *via* a video camera (JVC TK-1280E, Victor Company, Japan).

Modifications were made to the experiment described above in order to permit simultaneous SICM and SNOM imaging. Laser light (Laser 2000 Ltd., UK) of wavelength, 532 nm, was coupled *via* a multi-mode fibre (FG-200-UCR; 3M Specialty Optical Fibers, West Haven, USA) into the micropipette. In order to confine light of the aperture, 100-150 nm of aluminium was evaporated onto the walls of the pipette. The scattered laser light was collected by a x60 long working distance objective and relayed by transfer optics onto a PMT (D-104-814, Photon Technology International, Surbiton, England) to record the optical signal. During raster scan, this signal was recorded on the data acquisition computer, *via* an ADC, which also recorded the z position of the sample to obtain simultaneous optical and topographic images of the sample using the control/data acquisition hardware and software produced by East Coast Scientific (Cambridge, UK). The micropipette is shown in Figure 1.

Adult rabbit myocytes were isolated using a low calcium solution (NaCl 120, KCl 5.4, $MgSO_4$ 5, pyruvate 5, glucose 20, taurine 20, HEPES 10 and nitrotriacetic acid (NTA) 5 (mmol/L), preoxygenated with 100% O2) and collagenase and protease

enzymes as previously described (Jones *et al*, 1990). Cells were imaged on a glass coverslip in a low calcium medium at room temperature.

Optical and SICM images were recorded simultaneously and it took about 20 minutes to record one set of images. The micropipette used is estimated, by the measured ion current, to have an internal diameter of about 500 nm and was held about 250 nm over the surface during imaging. The estimate external diameter is 1000 nm, and comprises the glass and metal coating. This means that these images were recorded in the near field, less than a wavelength of light from the sample, with an aperture having a diameter comparable to the wavelength of light.

A 20 x 20 μm scan of the surface of a living rabbit cardiac myocyte showed that the sarcomeric structure running from bottom left to top right is clearly visible in both the SICM and SNOM image. The optical image appears to be generated only at the surface of the cell as expected using a scanning probe technique. The sarcomeres are visible in both images, although clearer in the optical image. In a large scan range, there was excellent correspondence between the optical and SICM images. The estimated resolution was about 500 nm.

CLAIMS

1. Apparatus for imaging an object, comprising a probe via which an assay component may be delivered; a sensor to detect ion current; and means for controlling the position of the probe relative to the object in response to the ion current.

2. Apparatus according to claim 1, wherein the probe is a micropipette.

3. Apparatus according to claim 1 or claim 2, wherein the assay component is light.

4. Apparatus according to claim 3, wherein the probe comprises a fibre optic.

5. Apparatus according to claim 3 or claim 4, which additionally comprises a laser light source.

6. Apparatus according to any of claims 3 to 5, wherein the probe contains a light-activatable dye at its tip.

7. Apparatus according to any of claims 3 to 6, wherein the outer surface of the probe is coated, e.g. with a metal layer, to prevent leakage of light.

8. Apparatus according to claim 1 or claim 2, wherein the probe contains, as the assay component, a substance that, at the surface of a live cell, produces a detectable change.

9. Apparatus according to claim 8, wherein said substance generates fluorescence, bioluminescence or chemiluminescence.

10. Apparatus according to claim 1 or claim 2, wherein the probe contains, as the assay component, a substance that, on delivery to a live cell, produces a detectable change inside the cell.

11. Apparatus according to any preceding claim, wherein the controlling means comprises means for vibrating the probe substantially normal to the surface of the object, and means for modulating the ion current.

12. A method for imaging an object in a liquid environment, by scanning ion-conductance microscopy, using a probe whose distance from the object is maintained in response to the ion current in the liquid, wherein the probe includes means for delivering an assay component to the object.

13. A method according to claim 12, wherein the probe and/or the assay component is as defined in any of claims 1 to 10.

14. A method according to claim 12 or claim 13, which comprises generating light and wherein the said distance is less than the wavelength of the light.

15. A method according to any of claims 12 to 14, wherein the probe is vibrated substantially normal to the surface of the object, and the said distance is controlled by modulation of the ion current.

16. A method according to any of claims 12 to 15, wherein the object is a live cell.

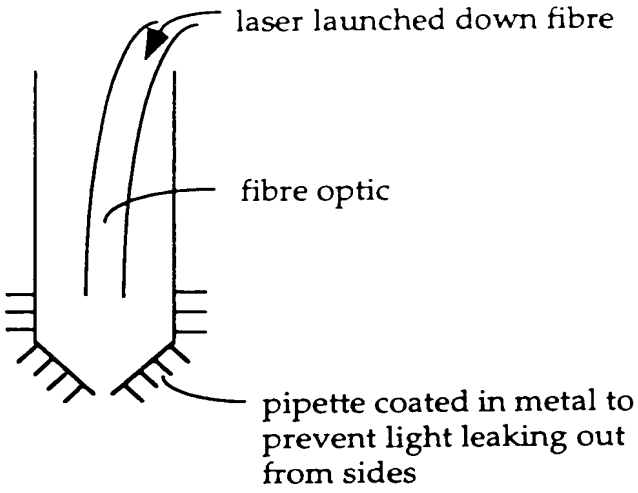
laser launched down fibre
fibre optic
pipette coated in metal to prevent light leaking out from sides

FIGURE 1

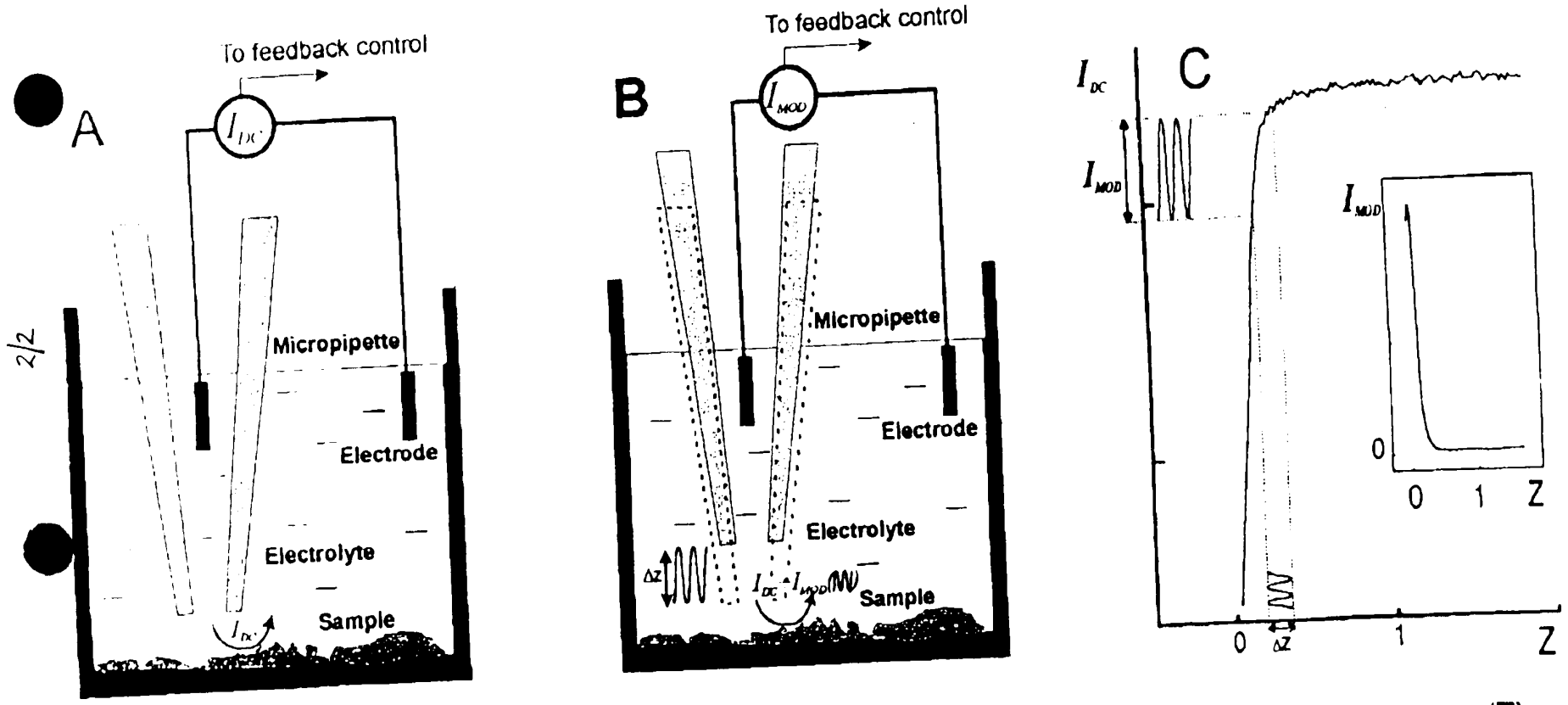

Figure 1. DC (I_{DC}) & modulated (I_{MOD}) tip current as a function of the sample/tip separation (Z)